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The microscopical characterization of membranes poly (L-glycolic-co-lactic acid) with and without added plasticizer: an in vivo study

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Abstract The development of biodegradable materials has lead to renewed interest in the study of their interactions with the host organism in order to make the resulting products appropriate for use as temporary materials in clinical research, as well as important therapeutic applications. The copolymer poly (L-lactic-co-glycolic acid) or PLGA membranes have been used for several purposes. The physical properties of these materials can be modified by the addition of a plasticizer, such as the triethylcitrate, to provide flexibility and porosity to the implants, and enhance control of the polymer degradation time. Membranes with 7% plasticizer and without plasticizer (triethylcitrate) were compared. Membranes without plasticizer were denser and more compact than those with plasticizer. Two days and 30 days after implantation, the membranes with and without plasticizer showed little degradation. Sixty days and 120 days after implantation, the membranes with 7% plasticizer showed more cell invasion, and tissue adherence, as well as rapid degradation when compared to membranes without plasticizer.

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Introduction

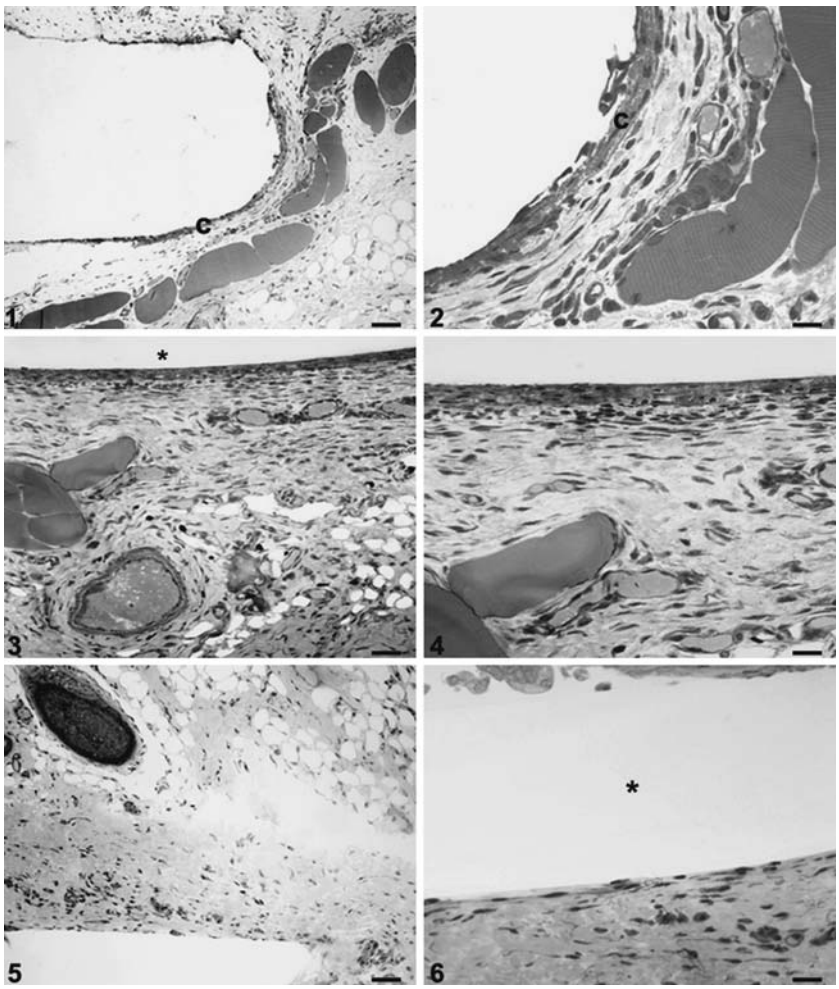
Biodegradable materials have various important applications in the biomedical field. There are basically two groups of polyesters which have significant importance: polylactides and polyhydroxybutyrates. Both groups degrade via hydrolysis with the rates of degradation depending on medium properties such as pH, temperature, solvent and presence of biocatalysts, as well as on chemical compositions. A number of the most popular polymers are of synthetic origin like teflon, nylon, polystyrene, etc. [1–3].

One of the major distinctions between the two groups is their degradability. The biological polymers, due to their synthesis via the enzymatic route, degrade rapidly in the biological medium. Since the synthetic organic polymers are produced via chemical synthesis, enzymes or microorganisms that degrade or utilize them have not evolved yet resulting in their slower disappearance from the environment [1, 4].

Membranes obtained by solution processing of poly (α-hydroxy acids) have found applications as supports for fixation and cell growth, as well as, drug delivery systems, vascular grafts, biodegradable skin substitutes and wound coverings. For these applications, the membranes should be porous, with interconnections between the pores [5–9].

Schugens et al. [10] synthesized a biodegradable implant of poly (lactic acid) with a controlled porosity which consisted of an aggregate of poly (L-D,L lactic acid) (PDLLA) spheres of known size. In addition, triethylcitrate, a biocompatible plasticizer acted on the polymer chains to reduce the interaction among them, thus favoring a flexible membrane [10–12].

In this report, we describe the biological and morphological properties of a porous membrane of PLGA con-



Figs. 1–6 **Fig. 1** PLGA membrane without plasticizer after 2 days of implantation. Note the capsule with collagen tissue **(c)** around the membrane. Glycol methacrylate, toluidine blue. Bar = 100 μm, **Fig. 2** PLGA membrane without plasticizer after 2 days of implantation. Note the connective tissue capsule **(c).** Glycol methacrylate, toluidine blue. Bar = 20 μm, **Fig. 3** PLGA membrane with 7% plasticizer after 2 days of implantation. Note the connective tissue fibers around the membrane **(*)**. Glycol methacrylate, toluidine blue. Bar = 100 μm, **Fig. 4** PLGA membrane with 7% plasticizer after 2 days of implantation. Glycol methacrylate, toluidine blue. Bar = 50 μm, **Fig. 5** PLGA membrane without plasticizer after 30 days of implantation. Note the dense connective tissue without adhesion of the cells on the membrane. Glycol methacrylate, toluidine blue. Bar = 100 μm, **Fig. 6** PLGA membrane without plasticizer after 30 days of implantation. Note the connective tissue surrounding the membrane **(*)**. Glycol methacrylate, toluidine blue. Bar = 50 μm

taining 7% triethylcitrate after implantation in the subcutaneous tissue of rats.

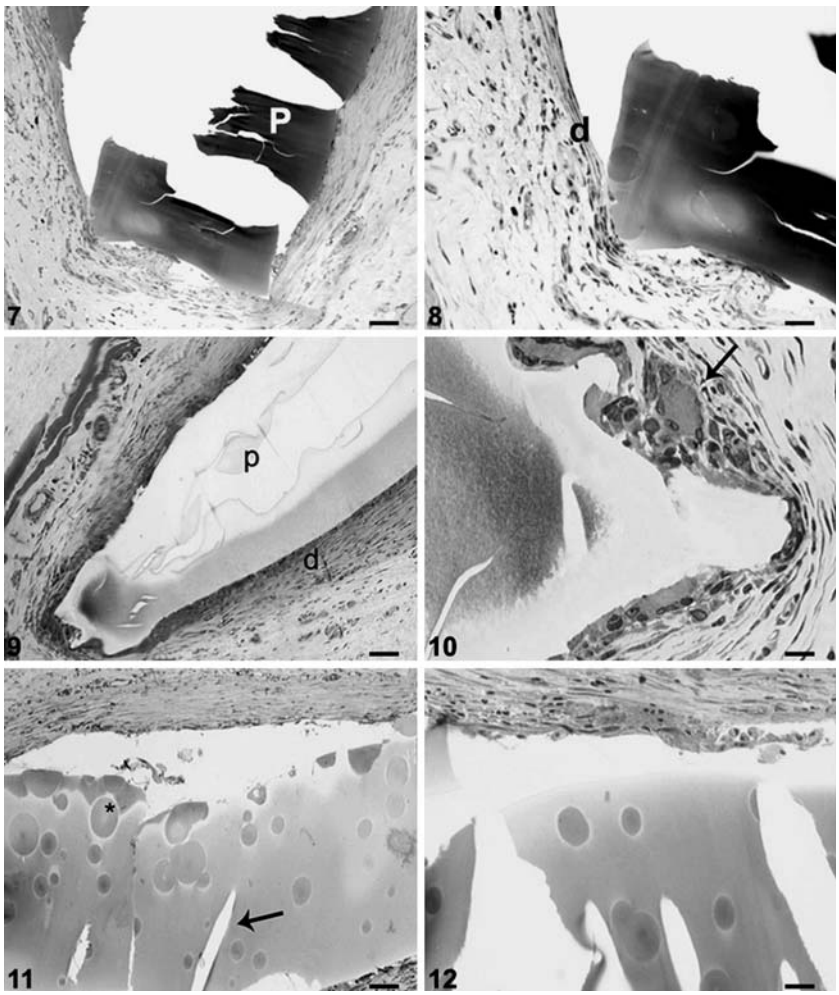
Materials and methods

Production of implants

PLGA was provided as pellets by PURAC (Groninghen, The Netherlands). About 10 g of polymer was dissolved in 100 ml of methylene chloride (CH_2Cl_2, Merck) containing 7% triethylcitrate (Aldrich) in a closed recipient at room temperature [11]. Other membranes were prepared without triethyltcitrate. The mixture was then poured onto a glass plate (100 cm^2) that was air dried (air flow of 1 l/min) at room temperature. After 15 h, the membrane was removed from the plate and vacuum dried for 24 h. Disks 5 mm in diameter and 620 μm thick were cut and used in the studies described below.

Implantation

The membranes were immersed in 70% ethanol and then vacuum dried. Sixteen female Wistar rats 3 months old obtained from university's central animal house were used. The rats were housed at 22 ± 2 °C on a 12 h light/dark cycle with food and water ad libitum. Two membranes were implanted in the dorsal subcutaneous tissue of rats anaesthetized with ketamine and xylazine-HCl (16.6 and 3.33 mg/kg, i.p., respectively) (Virbac, Brazil). Tissue samples were



Figs. 7–12 **Fig. 7** PLGA membrane with 7% plasticizer after 30 days of implantation. Note the connective tissue surrounding the polymer fragments **(P).** Glycol methacrylate, toluidine blue. Bar = 100 μm, **Fig. 8** PLGA membrane with 7% plasticizer after 30 days of implantation. Note the capsule collagen fibers **(d)** around the polymer. Glycol methacrylate, toluidine blue. Bar = 50 μm,**Fig. 9** PLGA membrane without plasticizer after 60 days of implantation. Note the connective tissue capsule **(d)** surrounding the polymer **(p)** and the degrading polymer. Glycol methacrylate, toluidine blue. Bar = 50 μm, **Fig. 10** PLGA membrane without plasticizer after 60 days of implantation. Note the capsule surrounding the membrane and the cellular infiltration with giant cells **(arrow)**. Glycol methacrylate, toluidine blue. Bar = 20 μm, **Fig. 11** PLGA membrane with 7% plasticizer after 60 days of implantation. Note the degrading polymer with fractures **(arrow)** and particles (*). Glycol methacrylate, toluidine blue. Bar = 100 μm, **Fig. 12** PLGA membrane with 7% plasticizer after 60 days of implantation. Note the details of the previous figure. Glycol methacrylate, toluidine blue. Bar = 50 μm

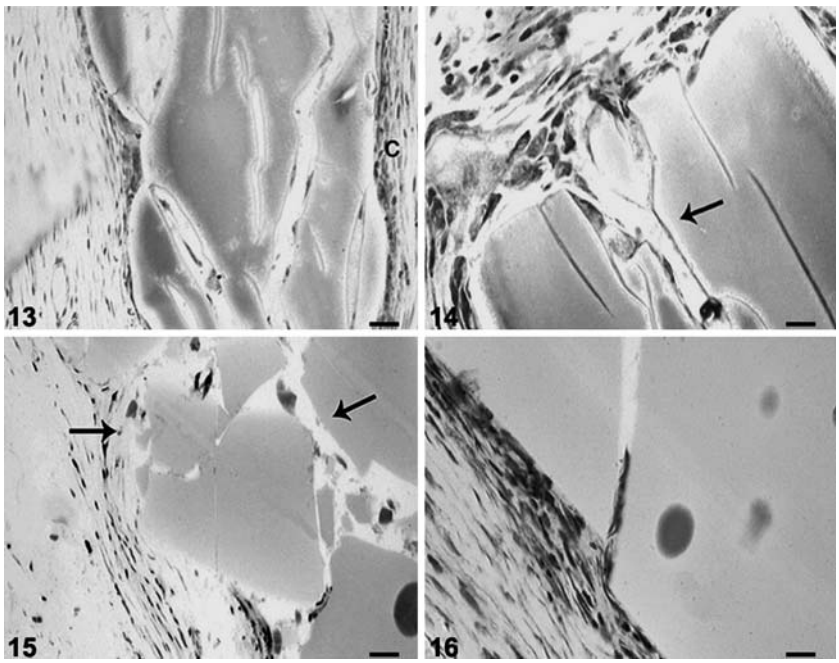
obtained 2, 30, 60 and 120 days post-implantation, after the rats were anaesthetized, and sacrificed.

Light microscopy

Fragments of skin were fixed in Bouin solution pH 7.4 (buffer solution) for 24 h at 4 °C, and embedded in paraffin. Sections of 5 μm thick were stained with Masson's trichrome method and Sirius red. Membrane fragments that had adhered to adjacent tissue were fixed in 4% formaldehyde and embedded in glycol methacrylate. Sections of 2 μm thick were stained with toluidine blue. These samples were observed, and photographed with a Nikon Eclipse E800 photomicroscope. The samples stained with Sirius red were observed, and photographed under polarized light to assess invasion by collagen fibers.

Transmission electron microscopy (TEM)

The samples were fixed in 2% glutaraldehyde, 2% formaldehyde, and 0.5% tannic acid 0.1 M phosphate buffer pH 7.4, for 3 h at 4 °C, followed by fixation in 1% OsO_4 for 1 h at room temperature. The samples were then embedded in Epon resin and uranyl acetate/lead citrate stained sections 60 nm thick were examined in a LEO Zeiss transmission electron microscope.



Figs. 13–16 **Fig. 13** PLGA membrane without plasticizer after 120 days of implantation. Note the connective tissue capsule **(c)** surrounding the polymer and cells inside the membrane. Glycol methacrylate, toluidine blue. Bar = 50 μm, **Fig. 14** PLGA membrane without plasticizer after 120 days of implantation. Note the details of the invasion tissue. Glycol methacrylate, toluidine blue. Bar = 20 μm, **Fig. 15** PLGA membrane with 7% plasticizer after 120 days of implantation. Note the fractures **(arrows)** in the degrading polymer. Glycol methacrylate, toluidine blue. Bar = 20 μm, **Fig. 16** PLGA with 7% plasticizer after 120 days of implantation. Note the fractures (arrows) in the membrane and the infiltration of the connective tissue. Glycol methacrylate, toluidine blue. Bar = 20 μm

Results

Macroscopic observation of the samples with and without plasticizer showed that there was no adhesion of the tissue to the membranes during the first 30 days after implantation. At intervals beyond this period, the sample was incorporated into the host tissue such that it was often difficult to locate the implant macroscopically in the skin at the site implantation.

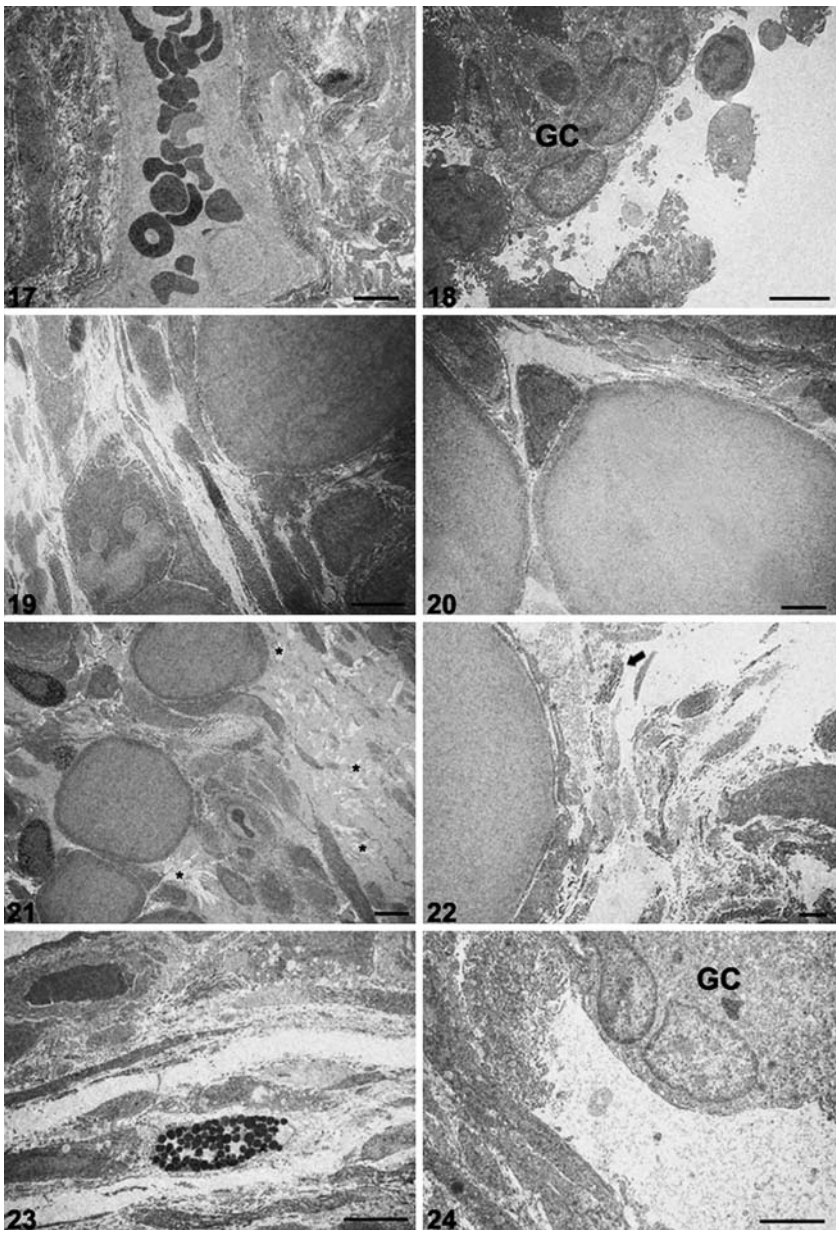
Microscopic analysis

In samples obtained early after implantation (2 and 30 days), there was a separation between the membrane and the adjacent tissue. However, after longer periods of implantation (60 and 10 days) was surrounded by adjacent tissue in the subcutaneous compartment.

About 2 days after implantation, the samples with and without plasticizer showed a massive infiltrate of polymorphonuclear cells in the membranes and a massive infiltration of polymorphonuclear cells embedded in a fibrin net, as well as numerous fibroblasts and macrophages. Samples with 2 and 30 days after implantation, there was no invasion of the membrane by connective tissue elements (Figs. 1–6,7–12). Sixty days after implantation, in the membranes without plasticizer, there was no membrane preservation and no inflammatory reaction (Figs. 7–12). By the other hand, the tissue around the membranes with plasticizer contained numerous macrophages. In this period, the membranes were no remarkable degradation (although slightly greater than in previous intervals), and no pores (Figs. 7–12). A connective tissue capsule was present. About 120 days after implantation, in membranes without plasticizer there was extensive tissue invasion and degradation was seen in membranes. Degradation resulted in the release of globular units from the membrane. The polymer was broken into small fragments surrounded by a thin network of connective tissue, with giant cells surrounding the membrane fragments (Figs. 13–16). In membranes with plasticizer, the invasion was less pronounced and there was less fragmentation. Cells were seen adhered to the membrane and the capsule contained a large number of collagen fibers (Figs. 13–16).

Transmission electron microscopy (TEM)

The examination of the samples with 2, 30, and 60 days after implantation, with and without plasticizer, showed the presence of giant cells and fibrillar elements on the extracellular matrix. Various cell types (fibroblasts, macrophages and giant cells) and small caliber blood vessels were present in the membranes (Figs. 17–24).



Figs. 17–24 **Fig. 17** TEM of PLGA membrane without plasticizer after 30 days of implantation. Note the blood vessels of the tissue that surrounded the polymer. Bar = 7 μm, **Fig. 18** TEM of PLGA membrane without plasticizer after 30 days of implantation. Note giant cell **(GC).** Bar = 7 μm, **Fig. 19** TEM of PLGA membrane with 7% plasticizer 30 days after implantation. Note the presence of cells and extracellular matrix components in the membrane implantation site. Bar = 7 μm, **Fig. 20** TEM of PLGA membrane with 7% plasticizer after 30 days of implantation. Note the connective tissue elements. Bar = 7 μm, **Fig. 21** TEM of PLGA membrane without plasticizer after 60 days of implantation. Note the fragments of polymer **(*)** and the invasion of connective tissue in this site. Bar = 7 μm, **Fig. 22** TEM of PLGA membrane without plasticizer after 60 days of implantation. Note the collagens fibers **(arrow)** in connective tissue. Bar = 3 μm, **Fig. 23** TEM of PLGA membrane with 7% plasticizer after 60 days of implantation. Note the massive presence of connective tissue elements. Bar = 7 μm, **Fig. 24** TEM of PLGA membrane with 7% plasticizer after 60 days of implantation. Note the presence of giant cell **(GC)** in site of implantation. Bar = 7 μm

Discussion

Triethylcitrate was selected as a water-soluble model plasticizer to study the effect of the changes of the plasticizer on the physic mechanical properties [12–14]. The drug (triethylcitrate) can be released by diffusion through water-filled pores or through the polymer and after hydrolytic degradation/erosion of the polymer. In addition,

the triethylcitrate provide certain flexibility to tubular implants in order to allow the application of sutures to the implant surface [15, 16].

The inclusion of the plasticizer significantly increased the flexibility of PLGA polymers. The addition of 7% plasticizer to PLGA induced a dense polymer formation, without pores, resulting in the small tissue invasion, as well as in the membranes without plasticizer analyzed by light microscopy.

For PLGA with plasticizer, the initial burst was followed by a lag phase of about 30 days and then an accelerated release was observed from the 30th day till completion of the release by 120th day. When compared with the PLGA without plasticizer, this degradation occurred more slowly.

Analysis of the samples 2 days after the implantation induced an inflammatory reaction with many neutrophils and macrophages. Local trauma related to the surgical procedures provoked a strong inflammatory reaction for up to 7 days after the implantation. After this period, the strong inflammatory response gave place to a reaction against the implant [17, 18].

As shown here, membranes with and without plasticizer were covered with a capsule of connective tissue. The surface of membranes without plasticizer remained smooth since it did not allow cell invasion, while the membrane containing plasticizer had a rough morphology that allowed cell growth on its surface, and was totally invaded by macrophages, fibroblasts and others elements of connective tissue [5].

Silva et al. [19] studied synthetic degradable implants of poly (lactic acid) with addition of 10% of triethylcitrate, resulting in porous membranes and confered malleability, which allowed the membranes to adapt to the movement and flexibility of soft tissue. They observed that membranes without plasticizer showed less adhesion of the host tissue to the implant material, and degraded more slowly. Low porosity membranes were suitable to induce tissue regeneration, whereas high porosity ones were useful in tissue reconstruction since they allowed better cell adhesion, and migration [19].

The degradation of membranes containing 7% plasticizer was faster than the observed in membranes without plasticizer. Membranes with 7% plasticizer showed high degradation about 60 days after implant action while in membranes without plasticizer this degradation occurred later.

TEM examination showed connective tissue invasion throughout the pores. Various cell types (fibroblasts, macrophages, and giant cells), small caliber blood vessels, and extracellular matrix were present in the membrane.

As shown here, 60 days after implantation, the membranes containing plasticizer were totally invaded, indicating that degradation is faster and cellular growth was more accentuated when compared to the membranes without plasticizer. The presence of blood vessels in the samples after 30 days implantation indicated a neovascularization process, which facilitated the regeneration of the damaged tissue.

Based on the results, the presence of plasticizer influences the porosity of PLGA, allowing the control of the degradation time and of the extension of the cellular invasion, which are important factors to the implant maintenance in host tissue.

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